

The NFIII/OCT-1 Binding Site Stimulates Adenovirus DNA Replication In Vivo and Is Functionally Redundant with Adjacent Sequences

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The inverted terminal repeat (ITR) of adenovirus type 5 (Ad5) is 103 bp in length and contains the origin of DNA replication. Cellular transcription factors NFI/CTF and NFIII/OCT-1 bind to sites within the ITR and participate in the initiation of viral DNA replication in vitro. The ITR also contains multiple copies of two conserved sequence motifs that bind the cellular transcription factors SP1 and ATF. We have analyzed a series of viruses that carry deletions at the left terminus of Ad5. A virus carrying a deletion of the NFIII/OCT-1, SP1, and ATF sites within the ITR (mutant *dl309-44/107*) was wild type for virus growth. However, the deletion of these elements in addition to sequences immediately flanking the ITR (mutant *dl309-44/195*) resulted in a virus that grew poorly. The analysis of growth parameters of these and other mutants demonstrate that the NFIII/OCT-1 and adjacent SP1 sites augment the accumulation of viral DNA following infection. The function of these elements was most evident in coinfections with a wild-type virus, suggesting that these sites enhance the ability of a limiting *trans*-acting factor(s), that stimulates viral DNA replication, to interact with the ITR. The results of these analyses indicate functional redundancy between different transcription elements at the left terminus of the Ad5 genome and demonstrate that the NFIII/OCT-1 site and adjacent SP1 site, previously thought to be nonessential for adenovirus growth, play a role in viral DNA replication in vivo.

Adenovirus DNA replication requires three viral gene products: the adenovirus DNA polymerase (Ad Pol), preterminal protein (pTP), and the single-stranded-DNA-binding protein (DBP) (reviewed in references 4, 42, and 47). Terminal sequence integrity of the linear genome is maintained by using pTP as a protein primer for DNA replication. pTP remains covalently attached to the 5' ends of the replicated DNA and is proteolytically processed to the mature form late in infection. Adenovirus replication origins are located at the ends of the genome within the inverted terminal repeats (ITRs). The adenovirus type 5 (Ad5) ITR is 103 bp in length (Fig. 1); the ITR is 102 bp in Ad2 and is nearly identical in sequence to the Ad5 ITR. In vitro studies using Ad5 and Ad2 have assigned the replication origin to sequences within the terminal 50 bp (reviewed in references 4, 42). The terminal 18 bp include conserved core sequences between nucleotides (nt) 9 and 18 that direct low levels of initiation in vitro. This minimal origin binds the pTP-Ad Pol complex (31, 46) and the cellular factor ORP-A (38). Maximal origin function in vitro requires the binding sites for the cellular factors NFI and NFIII located between nt 19 and 50 (Fig. 1) (reviewed in references 4, 42, and 47). These factors bind noncooperatively to their corresponding sites and stimulate the initiation of replication by different mechanisms (32). NFI DNA binding is stimulated by DBP (7, 45). NFI attracts the pTP-Ad Pol replication complex to the origin and stabilizes its interaction with this region via direct protein-protein interactions between NFI and Ad Pol (2, 5, 31, 32). The mechanism of enhancement by NFIII is not clear, although a role for the bending of DNA induced by NFIII binding has been postulated (48). In addition to their roles in

adenovirus DNA replication, cellular factors NFI and NFIII are also known to regulate the transcription of viral and cellular genes; NFI is related to transcription factor CTF (25), and NFIII is related to the transcription factor OCT-1 (33, 36).

In addition to DNA replication, *cis*-acting sequences at the left end of the adenovirus genome direct other events important for viral growth. The E1A enhancer region, located between nt 195 and 355, is required to stimulate immediate early transcription of region E1A and is composed of two distinct enhancer elements (21, 22). This region also contains sequences that are required for packaging of viral DNA late in infection (12, 13, 20). The ITRs of all human and simian adenoviruses contain two conserved sequences: a GC-rich motif and the sequence 5'-TGACGT-3' (40, 43). While different DNA-binding activities that recognize these sequence motifs have been identified, these elements in Ad5 have been shown to bind the cellular transcription factors SP1 and ATF (26, 30), respectively, and will be referred to here as the SP1 and ATF motifs. The adenovirus ITR possesses promoter and enhancer activities dependent on these conserved elements (18, 30, 34). Similar sequences are found between the ITR and the E1A enhancer (nt 104 to 194; Fig. 1). This region also contains binding sites for the cellular enhancer factors EF-1A (3) and EBP-1 (1) and has been shown to confer enhancer activity (23). It is likely that the transcriptional activity of the terminal 195 bp along with the E1A enhancer is involved in the E1A transcriptional start sites observed late in infection which initiate as far upstream as nt 125 (35, 39).

In this report, we examine the phenotype of viral mutants carrying deletions within and flanking the Ad5 ITR. Our results demonstrate that the NFIII/OCT-1 site and adjacent SP1 site, previously thought to be nonessential for virus growth, play a role in viral DNA replication in vivo. The

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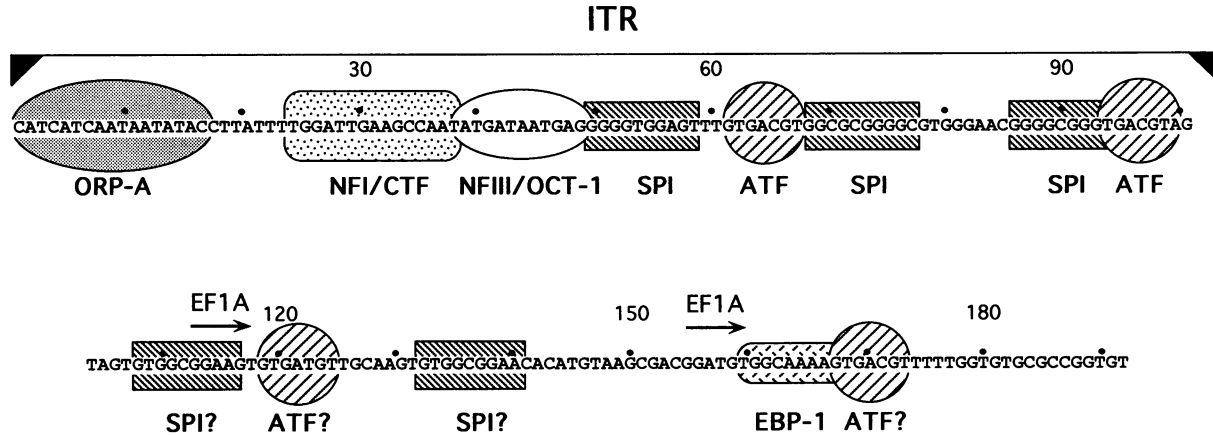


FIG. 1. The left-terminal 194 bp of Ad5. The nucleotide sequence of the Ad5 ITR (terminal 103 bp, indicated by a bracket above the sequence) and the left-end flanking sequences are shown. Numbers above the sequence represent nucleotide positions relative to the genomic terminus at nt 1. The stippled oval designates the region protected from DNase I by ORP-A (38). Recognition sequences for cellular factors NFI/CTF, NFIII/OCT-1, SPI, ATF, EF-1A, and EBP-1 (1, 3, 25, 26, 30, 33, 36) are indicated. Question marks indicate that binding of SPI or ATF to these binding site homologies has not been determined.

functional role of the NFIII/OCT-1 site could be replaced by sequences immediately flanking the ITR, suggesting a functional redundancy of these distinct sequences. The phenotype of viruses carrying mutations in the ITR was most evident in coinfections with a wild-type virus, indicating that the NFIII site, SPI site, and elements flanking the ITR enhance the ability of a limiting *trans*-acting factor(s), required to augment viral DNA replication, to interact with the adenovirus terminal repeat. These results suggest that different transcription factors may stimulate the interaction of such a component with the replication machinery.

MATERIALS AND METHODS

Plasmids and virus constructions. Plasmids used for virus constructions were derived from pE1A-WT, which contains the left-terminal 1,339 bp of the Ad5 genome cloned into pBR322 (21). A unique 8-bp *Bgl*II linker was inserted in plasmid pE1A-WT at either the *Rsa*I site at nt 194 or the *Hph*I site at nt 106, generating plasmids pEBL194 and pEBL106, respectively, which served as parental plasmids for deletion mutagenesis. Unidirectional deletions that originated at the *Bgl*II linker sites and progressed leftward toward the ITR were constructed by standard cloning procedures using exonuclease III and nuclease S1. An 8-bp *Bgl*II linker was inserted at each deletion endpoint. The extent of the deletion in each mutant plasmid was determined by nucleotide sequence analysis. The nomenclature of the plasmids reflects the last nucleotide position remaining at either side of the deletion junctions; e.g., deletion mutant pEB44/195 retains nt 44 and 195. Plasmids used to rebuild viruses *dl*309-44/195+27 and *dl*309-44/195+90 were constructed by inserting DNA fragments from different sources into the *Bgl*II linker site of pEB44/195. pEB44/195+27 contains an insertion of a 27-bp synthetic oligonucleotide carrying the bacteriophage T7 promoter (5'-GATCTCGC CCTATAGTGAGTCGTATTA-3'). pEB44/195+90 carries an insertion of a 90-bp fragment that contains five 17-mer GAL4 binding sites [*Pst*I-*Xba*I fragment from plasmid p(GAL4)₅/E1BCAT (27)]. All mutations constructed in plasmids were rebuilt into intact viruses by the method of Stow (44). Virus isolates were screened by restriction endonucle-

ase analysis of viral DNA, and the mutations of the final virus particle stocks were confirmed by ³²P-labeling *Bgl*II restriction fragments with Klenow polymerase and sizing the left-end fragments in comparison with the parental plasmid fragment by electrophoresis on a 12% sequencing gel as previously described (19). Purified virus particles were prepared by CsCl equilibrium gradient sedimentation, titered by optical density (1 optical density unit at A₂₆₀ equals 10¹² particles), and used for all infections.

Cells, infections, and determination of virus yields. 293 (14) and HeLa cell lines were maintained as monolayers in Dulbecco modified Eagle medium containing 10% supplemented calf serum (HyClone). HeLa cells were also propagated in suspension in minimal essential medium containing 5% supplemented calf serum. Virus infections were performed with purified virions at a multiplicity of 200 particles per cell, except where otherwise indicated, at 37°C for 1 h. After infection of monolayers, cells were washed twice with phosphate-buffered saline (PBS) before the addition of fresh medium. Infected cell lysates were generated by three freeze-thaw cycles, and infectious virus yields were determined by plaque assays on 293 cells.

Viral DNA accumulation, replication rates, and packaging efficiency determination. For experiments involving mixed virus infections, HeLa suspension cells were coinfecting with both mutant and wild-type *dl*309 viruses as described above. To assay the accumulation of viral DNA, total nuclear DNA was isolated at 3, 8, 16, and 24 h postinfection (p.i.). Total nuclear DNA was isolated by lysing infected cells by the addition of Nonidet P-40 to 0.4%, harvesting the nuclei by centrifugation, and isolating DNA as described previously (28). The isolated DNA was digested with *Cla*I and *Bgl*II, to distinguish mutant from wild-type DNA, and analyzed by Southern hybridization (28) using pE1A-WT, ³²P-labeled by the random primer method (11), as a probe. Ratios of wild-type to mutant DNAs were determined by densitometric scanning of autoradiographs generated by using pre-flashed film. Viral DNA accumulation in the nucleus of single-virus-infected HeLa monolayer cells was determined by isolating total nuclear DNA at 3, 8, 12, and 16 h p.i. Viral DNA in samples of equal amounts of total nuclear DNA was quantitated by slot blot analysis (49) using a ³²P-labeled

pE1A-WT probe as described above. To confirm that equal amounts of total DNA were represented in all samples, aliquots were also analyzed with an actin-specific probe (data not shown). Two different dilutions of samples from a typical experiment were quantitated by liquid scintillation spectroscopy of the hybridized filter pieces, and values of virus-specific counts per minute were normalized to the input (3-h) value for each virus.

Viral DNA replication rates were determined by [^3H]thymidine incorporation into newly synthesized viral DNA. Infected cells were labeled at 8, 12, and 16 h p.i. for 1 h with 100 μCi of [^3H]thymidine per ml. Following the pulse, the cells were washed twice with PBS, and total nuclear DNA was isolated, digested with restriction endonuclease *Hind*III, fractionated on a 1% agarose gel, and fluorographed. Replication rates at 12 and 16 h p.i. were quantitated by densitometric scanning of virus-specific bands on autoradiograms from three independent experiments.

To determine the packaging efficiencies of mutant viruses, HeLa cells coinfecting with wild-type and mutant viruses were harvested at 24 or 48 h p.i., as indicated. Total nuclear DNA was isolated from one half of the cells, and virion DNA was isolated from the other half. Virion DNA was isolated as described previously (12). Briefly, virions were isolated from cleared lysates of infected cells lysed in 0.2% deoxycholate and 10% ethanol at pH 9 and then treated with RNase A and DNase I. Virus particles were lysed in 0.5% sarcosyl, and the viral DNA was digested with proteinase K, extracted extensively with phenol-chloroform, and precipitated with ethanol. Total nuclear and virion DNAs were analyzed by Southern hybridization as described above.

Detection of viral DBP protein levels. The levels of the E2a gene product DBP in virus-infected cells were determined by Western immunoblot analysis (17). Aliquots of infected cells used for viral DNA replication rate determination were harvested at 8, 12, and 16 h p.i., and whole cell extracts were prepared by radioimmunoprecipitation assay lysis as described previously (9). Protein concentrations were determined by using the Bio-Rad protein assay reagent (Bio-Rad Laboratories). A 50- μg amount of each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and assayed for relative amounts of DBP by using clone B6 anti-DBP monoclonal antibody (37), detected by chemiluminescence with use of ECL Western blotting detection reagents (Amersham).

RESULTS

Viruses with mutations in the NFIII site are defective for growth. A series of recombinant viruses carrying deletions which originated at the border of the E1A enhancer and packaging region at nt 194 and extended leftward toward the ITR was constructed (Fig. 2A). The growth of these mutant viruses relative to that of wild-type *dl309* was determined by a one-step growth curve on HeLa and 293 cells (Fig. 2B). These results showed that functionally redundant elements important for virus growth are present beyond the terminal 44 bp. Insertion of a *Bgl*II linker at nt 194 (*dl309*-EBL194) produced a phenotypically wild-type virus. Deletion of sequences between nt 76 and 195 (*dl309*-76/195) produced a slightly defective virus which grew to levels within twofold of the wild-type level. This virus, *dl309*-76/195, retains one copy each of the binding sites for cellular transcription factors NF1/CTF, NFIII/OCT-1, SP1, and ATF. Removal of the remaining ATF (*dl309*-60/195) and ATF and SP1 (*dl309*-53/195) elements produced viruses that also were slightly

defective for growth (levels three- to fivefold below the wild-type level). Mutant virus *dl309*-44/195 carries a deletion of sequences between nt 44 and 195 and retains only the NF1 binding site and core origin of replication. This virus grew very poorly and produced a high percentage of small plaques in both cell lines. Yields of *dl309*-44/195 were typically 30- to 50-fold below the wild-type level and varied within this range among experiments. We attribute this variability in yields to the inability to detect all of the very small plaques. An independent mutant isolate, *dl309*-44/195B, gave similar results. The defect observed with *dl309*-44/195 in comparison with *dl309*-53/195 defines a functional role for NFIII/OCT-1 in vivo. Deletion to nt 36 disrupted the remaining NF1/CTF site and was lethal in repeated reconstruction experiments. Insertion of a *Bgl*II linker at nt 106, or deletion of the NFIII/OCT-1, SP1, and ATF motifs within the ITR (sequences between nt 44 and 107) but with maintenance of the sequences flanking the ITR, produced a virus (*dl309*-EBL106 or *dl309*-44/107, respectively) which grew like the wild type (Table 1) (18, 19).

Viruses carrying insertions of variable size and sequence in the *dl309*-44/195 deletion background were constructed to confirm that the defect observed with *dl309*-44/195 was sequence specific and not due to a spacing effect (e.g., with respect to the packaging sequences). DNA fragments carrying either five binding sites for the yeast transcription factor GAL4 (90 bp) or the bacteriophage T7 promoter (27 bp) were inserted into the *Bgl*II restriction site of *dl309*-44/195. The resulting insertion virus (*dl309*-44/195+90 or *dl309*-44/195+27, respectively) retained the defective growth phenotype of the parental virus (Table 1).

The loss of sequences from the left end of the viral genome may affect growth of mutant virus *dl309*-44/195 by perturbing a number of viral processes; E1A gene expression, viral DNA replication, and viral DNA packaging are processes that are directed by left-end sequences. The adenovirus ITR contains intrinsic promoter and enhancer activities mediated primarily by the conserved SP1 and ATF motifs (18, 30, 34). Therefore, expression of E1A in the deletion viruses was examined. Both the steady-state levels of E1A transcripts and the utilization of upstream start sites late in infection (35, 39) were unaffected with mutant viruses *dl309*-76/195, -53/195, -44/195, and -44/106 (data not shown). In addition, the growth curves of the mutant viruses were the same in HeLa and 293 cells (Fig. 2B), which provide the E1A gene products in *trans*, suggesting that the defects observed were not at the level of E1A expression.

The NFIII/OCT-1 binding site augments viral DNA replication in vivo. DNA replication of the mutant viruses was examined in a coinfection assay. In this assay, HeLa cells were coinfecting at equal multiplicities of infection with a mutant virus and wild-type *dl309*, which served as a complementing virus for any defect in *trans* and as an internal control. Total nuclear DNA was harvested 3 h p.i., before the onset of viral DNA replication, to determine the input ratio of mutant to wild-type DNA, and at various time points after the onset of replication. Ratios of viral DNAs were determined by Southern hybridization in which mutant DNA was distinguished from *dl309* DNA by restriction endonuclease analysis. The accumulation of mutant relative to wild-type DNA was determined by comparing the ratios at various time points after replication to the input ratio. Equivalent amounts of mutant and *dl309* DNAs were present in the nucleus of coinfecting cells before the onset of viral DNA replication, as illustrated by the 3-h time point (Fig. 3). These input ratios indicated that the virus particle titers were

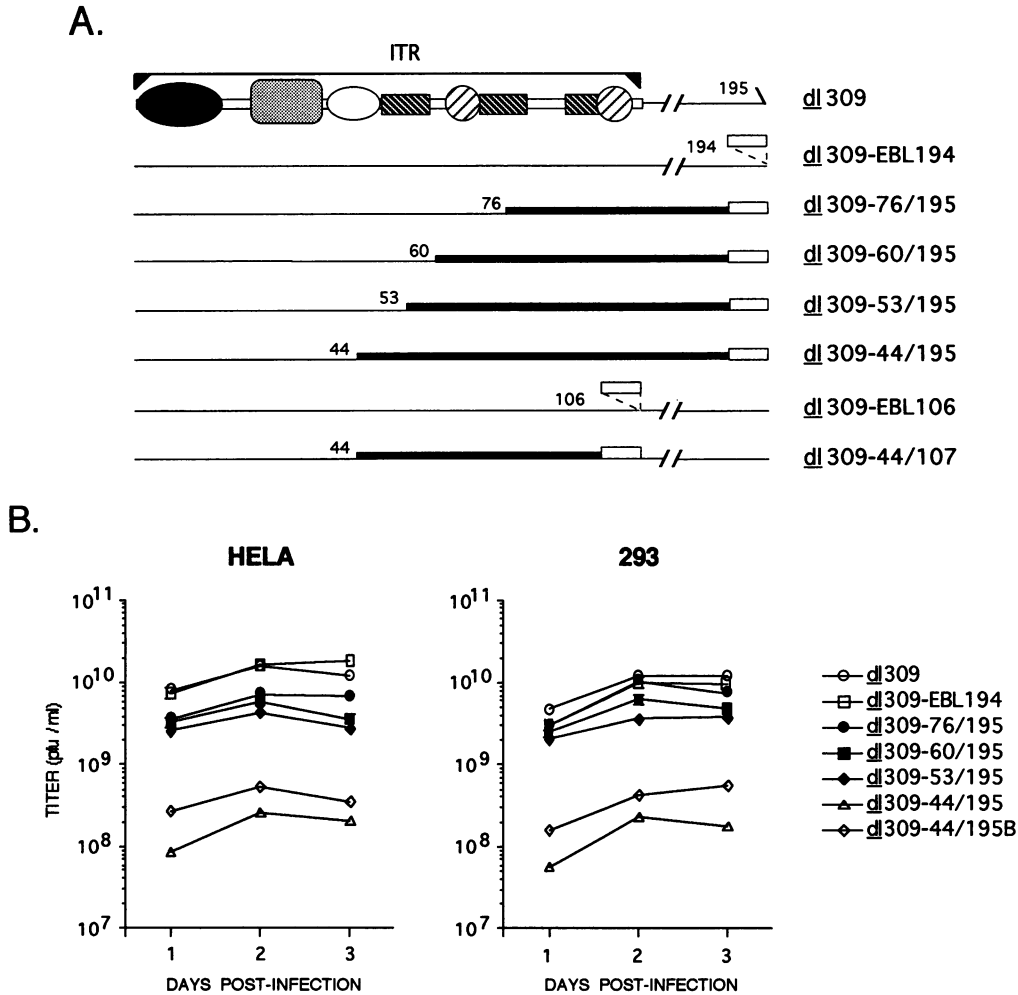


FIG. 2. (A) Schematic diagram of mutant viruses. The left-terminal 195 bp of wild-type virus *dl309* is shown schematically at the top. Binding sites within the ITR for cellular transcription factors are indicated as described in the legend to Fig. 1. Thick lines represent deleted sequences that have been replaced with *Bgl*III linkers (white rectangles). EBL194 and EBL106 signify that a *Bgl*III linker has been inserted after nt 194 and 106, respectively; the nomenclature for deletion viruses indicates nucleotide positions remaining at deletion junctions. (B) One-step growth curves on HeLa and 293 cells. HeLa and 293 cells were infected at a multiplicity of 200 particles per cell. Lysates were harvested at 1, 2, and 3 days p.i., and PFU titers were determined on 293 cells. Titers were determined twice in each of two independent experiments, and the averaged values are plotted. The one-step growth curves are distinguished by symbols indicated on the right.

correct and that all deletion viruses were able to enter the cells and deliver their DNA to the nucleus. Virus *dl309-EBL194* (as well as *dl309-44/107*; data not shown) produced the same ratio of wild-type to mutant DNA as the input ratio during the entire course of infection, thus exhibiting wild-

TABLE 1. Virus yields with spacing mutants

Virus	Yield (% of WT value) ^a
<i>dl309-44/195</i>	2.1
<i>dl309-44/195</i> +90 ^b	1.9
<i>dl309-44/195</i> +27 ^b	1.6
<i>dl309-EBL106</i>	100
<i>dl309-44/107</i>	45

^a Determined for lysates taken 2 days p.i. in two independent experiments and expressed as a percentage of the wild-type *dl309-EBL194* titer.

^b Recombinant virus constructed by the insertion of different-size DNA fragments into the *Bgl*III linker site of *dl309-44/195* as described in the text.

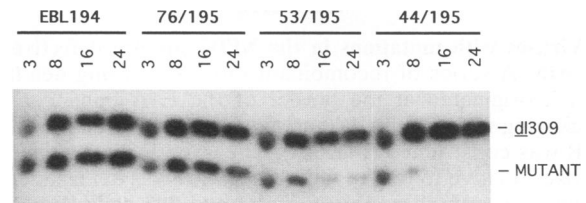


FIG. 3. Accumulation of mutant DNAs in cells coinfecting with a wild-type virus. HeLa cells were coinfecting with wild-type *dl309* and the indicated mutant viruses each at equal multiplicities. Total nuclear DNA was prepared at 3, 8, 16, and 24 h p.i. and digested with *Cla*I plus *Bgl*III. Various dilutions of the digested fragments were fractionated on a 1% agarose gel, and viral left-end fragments were detected by Southern hybridization using a probe containing left-end adenovirus DNA sequences. The migration positions of mutant and wild-type internal control *dl309* fragments are distinguished as indicated. More total DNA was loaded in the 3-h input samples in order to detect the viral DNA, causing these samples to run aberrantly.

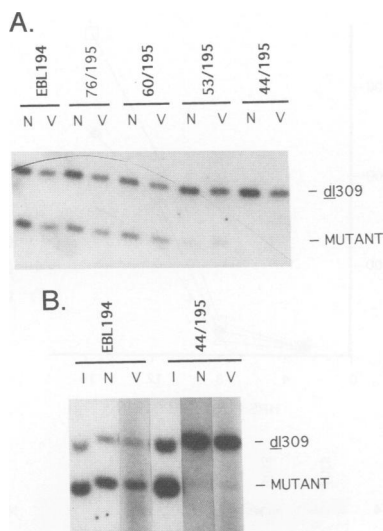


FIG. 4. Packaging efficiencies of mutant virus genomes. (A) Total nuclear (N) and virion (V) DNAs were isolated 48 h after coinfection of HeLa cells with wild-type *dl309* and the indicated mutant viruses. The samples were analyzed by Southern hybridization as described for Fig. 3. (B) HeLa cells were infected with either *dl309*-EBL194 or *dl309*-44/195 at a multiplicity fivefold higher than that used for the coinfecting wild-type *dl309*, harvested at 24 h p.i., and analyzed for packaging efficiencies as described for panel A. The input (I) high-molecular-weight nuclear DNA samples harvested at 3 h p.i. are included for comparison.

type kinetics of DNA replication. Nuclear DNA accumulation with mutant *dl309*-76/195 and -60/195 (data not shown) was within twofold of that of the coinfecting wild-type *dl309*. Mutant *dl309*-53/195 was clearly reduced for DNA accumulation relative to the wild-type virus. Mutant *dl309*-44/195 was severely defective for DNA accumulation (greater than 20-fold less efficient than *dl309*) in the coinfection assay. These results correlate the inability of a given mutant virus to grow well, as determined by a one-step growth curve with a defect in viral DNA replication, and show that the defect is *cis* acting, since essential gene products required for replication should be provided in *trans* by the coinfecting wild-type virus.

The left-terminal 380 bp of Ad5 are involved in viral DNA packaging (12, 13, 20). This observation prompted us to use a coinfection assay to test the efficiency with which the deletion mutant viruses packaged their DNA into virus particles (Fig. 4). Total nuclear DNA was harvested 48 h after coinfection of HeLa cells with wild-type and mutant viruses to determine the nuclear pool of mutant and wild-type DNA available for packaging. This determination was compared with the ratio of mutant to wild-type DNA that was productively packaged by analyzing virion DNA from virus particles isolated at the same time point (Fig. 4). The ratio of mutant to *dl309* DNA available in the nucleus was correctly reflected in the ratio of DNAs that were actually packaged into stable virions for all of the deletion mutant viruses, demonstrating that these viruses packaged their DNA with wild-type efficiencies. Because the mutant virus *dl309*-44/195 replicated so poorly, a fivefold-higher input ratio of mutant DNA relative to *dl309* was used in order to detect the mutant DNA after 24 h of infection (Fig. 4B). Unequal ratios of mutant relative to wild-type DNA in the

nucleus at the time of packaging did not bias the packaging efficiency of one DNA over the other (*dl309*-EBL194). These results showed that mutant *dl309*-44/195 packaged the low level of viral DNA that was available in the nuclear pool, demonstrating the absence of a *cis*-acting defect in packaging.

The NFIII/OCT-1 binding site stimulates the rate of viral DNA replication in vivo. The coinfection experiments demonstrated that for the mutant viruses which grew poorly (*dl309*-76/195, -60/195, -53/195, and -44/195), the kinetics of viral DNA accumulation were reduced relative to that of the coinfecting wild-type virus. We next investigated whether this defect reflected a decrease in the initiation of viral DNA replication by analyzing DNA replication rates. Singly infected HeLa cells were pulse-labeled for 1 h with [³H]thymidine at 8, 12, and 16 h p.i., and total nuclear DNA was harvested. Viral DNA was detected by fractionation of restriction endonuclease digests on an agarose gel and fluorography. The results of a typical DNA replication rate experiment are shown in Fig. 5A. At the onset of viral DNA replication (8 h p.i.), no or marginally detectable viral DNA was evident relative to the smear of labeled cellular DNA. At 12 h p.i., viral DNA as well as partial host DNA replication shutoff (reduction in background smearing) were evident. The relative DNA replication rates of *dl309*-53/195 and *dl309*-44/195 were approximately 2.5- and 4-fold below wild-type *dl309*-EBL194 levels. By 16 h p.i., the shutoff of cellular DNA replication was complete. The DNA replication of *dl309*-53/195 and *dl309*-44/195 had increased to rates equivalent to and twofold below that of the wild-type virus *dl309*, respectively. These data indicate that the mutant viruses were reduced for the initiation of replication and displayed a lag in the onset of DNA replication.

Figure 5B shows the results of a Western blot to analyze the amounts of viral DBP present in the same samples assayed for DNA replication rates in Fig. 5A. The results indicate that the relative amounts of early gene products required for viral DNA replication, as measured by DBP, were equivalent at the onset of viral DNA replication and were similar during the course of infection for each of the viruses.

To quantitate the overall DNA replication defect, viral DNA accumulation in the nucleus of cells infected with single mutant viruses was determined (Fig. 6). Viral DNA accumulation in HeLa cells was assayed by slot blot analysis of total nuclear DNA harvested at 3, 8, 12, or 16 h after infection with the wild-type and mutant viruses. Viral DNA accumulation was quantitated by liquid scintillation spectroscopy. The data are plotted in Fig. 6A; the results of a typical experiment are shown in Fig. 6B. Input levels of DNA (3 h) were similar in the *dl309*-53/195 and *dl309*-44/195 samples relative to the wild-type *dl309*-EBL194. Over the time course of the experiment, *dl309*-53/195 and *dl309*-44/195 DNA consistently accumulated to levels 1.5 and 2-fold, respectively, below that of wild-type *dl309*-EBL194 DNA. The reduction in mutant *dl309*-44/195 DNA accumulation was not due to DNA instability in the nucleus, since mutant viral DNA was found to be as stable as wild-type DNA as determined by pulse-chase experiments (data not shown). These results are consistent with the DNA replication rate results for these two viruses. The more severe extent of the *cis*-acting DNA replication defect observed with *dl309*-44/195 in the coinfection assays (Fig. 3 and 4) was likely exaggerated as a result of competition with the coinfecting wild-type *dl309* DNA for a limiting factor(s) required for DNA replication.

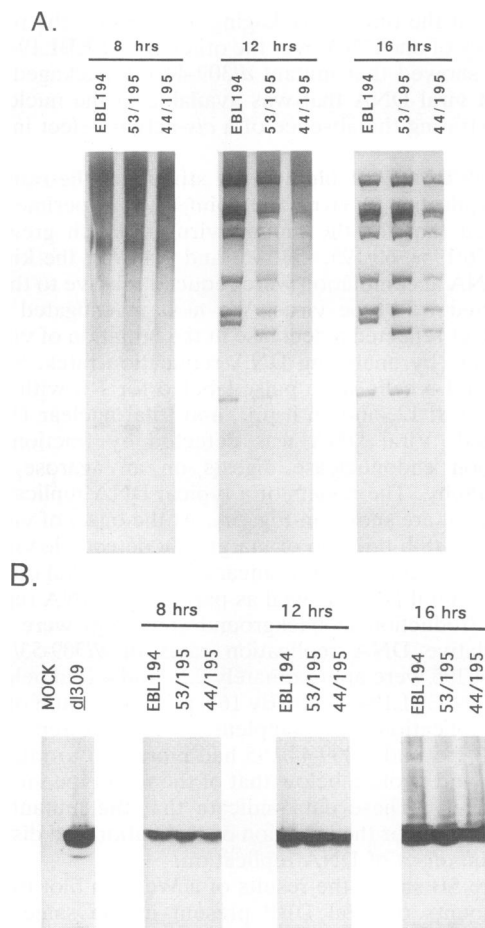


FIG. 5. Viral DNA replication rates. (A) Autoradiograph of the fluorographed gel from a typical experiment in which HeLa cells were infected with the indicated mutant viruses and pulse-labeled for 1 h with [3 H]thymidine at 8, 12, and 16 h p.i. Total nuclear DNA was harvested, and equal amounts of *Hind*III-digested total DNA were subjected to electrophoresis on a 1% agarose gel. (B) Viral DBP protein levels. Whole cell protein lysates were prepared at 8, 12, and 16 h p.i. from aliquots of the infected cells used for panel A. Viral DBP-specific bands were detected in samples of equal amounts of total protein by Western blotting using anti-DBP antiserum. Mock- and *dl309*-infected samples were included as controls.

DISCUSSION

The analysis of a series of viral mutants carrying deletions which impinge on the Ad5 ITR demonstrated that sequences previously believed to be nonessential for virus viability contributed in a significant manner to viral growth. Specifically, mutant virus *dl309-44/195*, which lacks the NFIII/OCT-1 binding site in the ITR and a series of adjacent, repeated SP1 and ATF binding motifs and the binding sites for other transcription factors (Fig. 1), was reduced 30- to 50-fold in yield, as assayed in a single-step growth curve (Fig. 2B). A second, independently constructed isolate, *dl309-44/195B*, displayed the same growth characteristics, making it unlikely that the defect was due to a fortuitous second-site mutation. Mutant viruses which maintained the NFIII/OCT-1 site but lacked the adjacent SP1 and ATF sites (*dl309-53/195*, -60/195 and -76/195) also displayed a discernible, although less defective, reduction in virus yield. The defect in growth in infections with *dl309-44/195* was not at

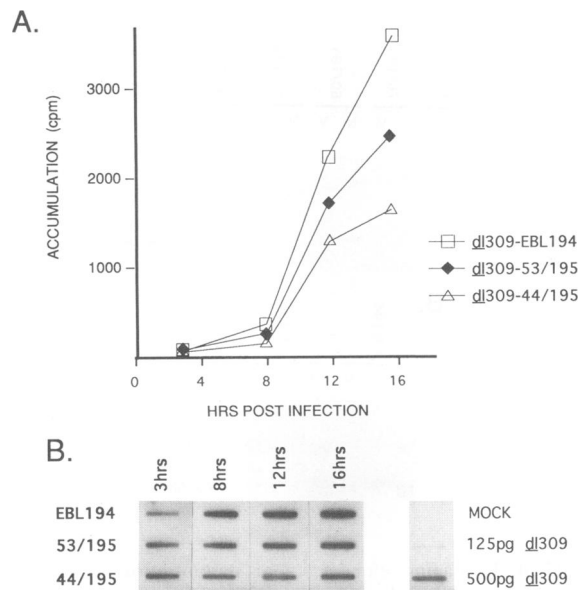


FIG. 6. Viral DNA accumulation in the nucleus of single-virus-infected cells. HeLa cells were infected with the indicated mutant viruses, and total nuclear DNA was prepared 3, 8, 12, and 16 h p.i. Samples containing equal amounts of total DNA were denatured, and a different dilution for each time point was slot blotted onto a nitrocellulose membrane. The amount of viral DNA in each sample was quantitated after Southern hybridization using a left-end adenovirus DNA probe by liquid scintillation spectroscopy of the filter sections. (A) Results for the experiment shown in panel B, plotted against the time p.i. that the sample was prepared. After adjustment for dilution factors, the amount of steady-state viral DNA was determined as virus-specific counts per minute in the original sample. (B) Autoradiograph of a typical DNA accumulation experiment, showing total DNA isolated at 3, 8, 12, and 16 h p.i. Controls included 1.5 μ g of a mock-infected sample and 125 and 500 pg of *dl309* DNA.

the level of appearance of viral DNA in the nucleus immediately after infection, representing the entry and uncoating process, since viral DNA accumulation in the nucleus prior to the onset of viral DNA replication was equivalent to the wild-type level in coinfection experiments (Fig. 3). The defect also did not reflect reduced E1A expression (Fig. 2B, HeLa versus 293, and data not shown) or a lack of any other virus-encoded *trans*-acting component, since the defect with mutant *dl309-44/195* was most evident in a coinfection with a wild-type virus, in which case all necessary products would be provided in *trans* (Fig. 3). The packaging of *dl309-44/195* DNA into virions was not deficient in coinfection assays (Fig. 4), indicating that this mutant retained sufficient packaging information in *cis*. Finally, the reduction in yield with *dl309-44/195* did not reflect a spacing effect due to the removal of DNA sequences; the insertion of 27 or 90 bp of heterologous DNA at the deletion junction with this mutant did not restore wild-type viability (Table 1).

The major defect with *dl309-44/195* was found to be at the level of the rate of viral DNA replication. This represents the first demonstration that the NFIII/OCT-1 site augments viral DNA replication *in vivo*, as has been described in reports of studies using *in vitro* replication assays. Coinfection experiments showed a dramatic reduction in the kinetics of viral DNA accumulation for *dl309-44/195* relative to the coinfecting wild-type virus *dl309* (Fig. 3 and 4). These results

showed that the DNA replication defect observed was *cis* acting. When viral DNA accumulation and replication rates were determined in single infections (Fig. 5 and 6), the results showed that both the DNA accumulation and replication rates of *dl309-44/195* were reduced but reached levels within two- to threefold of wild-type levels. The exaggerated magnitude of the DNA replication defect observed with *dl309-44/195* in the coinfection assay (greater than 20-fold) was likely due to competition with the coinfecting wild-type virus for a limiting *trans*-acting factor(s) required to stimulate viral DNA replication.

The growth of *dl309-44/195* was rescued by functionally redundant sequences located in the ITR flanking region between nt 106 and 195; mutant virus *dl309-44/107* and a similar virus (deletion of Ad2 sequences between nt 45 and 107) described by Hay and McDougall (19) each grew like the wild type. Redundancy was also evident between the terminus-distal half of the ITR and the ITR flanking region; deletion of sequences between either nt 44 and 106 or nt 106 and 195 produced viruses *dl309-44/107* and *dl309-6* (21), respectively, which grew like the wild type; however, removal of both blocks of sequences (nt 44 to 195) was detrimental for the resulting virus *dl309-44/195*. Sequences that are similar to the SP1 and ATF motifs, conserved in multiple copies in the terminus-distal half of the ITR, are evident in multiple copies in the ITR flanking region (Fig. 1). We cannot distinguish from these analyses whether these sites or other regulatory elements in this region contribute to virus growth. The data strongly indicate that different transcription elements contribute to the level of viral DNA replication. This functional redundancy may reflect a common mechanism of action.

How does a dramatic 30- to 50-fold decrease in *dl309-44/195* yields come about from a 2- to 3-fold decrease in viral DNA accumulation? Several points are worth considering. First, virus yields were determined by plaque assays, and it is possible that the variability in observed yields for *dl309-44/195* was due to the inability to detect the unusually high number of small plaques formed by this mutant. Although the growth of *dl309-44/195* was clearly defective, the yields may be artificially low because of small plaque size. Consistent with this idea is the fact that mutant *dl309-44/195* was reduced only 5-fold in virus yield in A549 cells (data not shown), compared with the ~50-fold reduction observed in 293 cells (Fig. 2B and Table 1). Larger viral plaques are produced with A549 cells than with 293 cells, allowing for more accurate quantitation of virus titers with this mutant virus. Additionally, the particle-to-PFU ratio for the wild-type virus was ~20 on 293 and A549 cells, while that of mutant *dl309-44/195* was ~200 on 293 cells and ~60 on A549 cells. Second, while postreplication steps in *dl309-44/195* virion production appeared normal (late viral protein synthesis was nearly at the wild-type level [data not shown], and no *cis*-acting packaging defect was observed when this mutant was coinfecting with wild-type virus [Fig. 4]), the possibility of a *trans*-acting defect in viral DNA packaging still remains. A small RNA has been identified as an essential component in the packaging of bacteriophage ϕ 29 (15, 16). This bacteriophage is similar to adenovirus in genetic structure and organization. The catalytic packaging sRNA of ϕ 29 is encoded by sequences at the left end of the linear double-stranded DNA genome (16) which, as in adenovirus, directs packaging. Thus, because of the transcriptional competence of Ad5 left-end sequences (18, 30, 34, 35), expression of such a putative packaging RNA may have been perturbed with mutant *dl309-44/195*, resulting in inefficient packaging in

singly infected cells. Third, although the levels of mutant virus DNA in infected cells approached that of the wild type (Fig. 5), a lag in the onset of replication or a reduction in the levels of viral DNA accumulation may disrupt the efficient progression of the virus life cycle. By analogy, adenoviruses with certain mutations in early region 4 display a lag in viral DNA accumulation, although wild-type levels are eventually reached, that results in small plaque size and lower virus yield (24, 50).

The NFI/CTF and NFIII/OCT-1 binding sites, as well as the regions which rescue the DNA replication defect of *dl309-44/195* (23, 39), function also as transcription elements. The use of transcription elements to augment or regulate origin function is not unprecedented (reviewed in reference 10), and a variety of mechanisms have been described. For instance, transcription elements can regulate origin function by forming a promoter. Active transcription in the vicinity of the *Escherichia coli* chromosomal origin (*oriC*) is required for initiation of DNA replication *in vivo* (reviewed in reference 51). In purified replication systems, such transcriptional activation is observed only when *oriC* melting by DnaA protein is inhibited by agents which stabilize duplex formation, e.g., high concentrations of the *E. coli* histone-like protein HU or low temperatures. Transcriptional activation in this case is mediated by an R loop, formed between the persistent RNA transcript and the template strand of DNA, which helps DnaA overcome topological constraints in unwinding (41). Similarly, transcriptional activation of the bacteriophage lambda origin is required *in vivo* and acts to overcome inhibition by HU *in vitro* (29). Alternatively, the site-specific DNA-binding activity of transcription factors can influence DNA replication initiation. Experiments suggesting that specific binding of NFI/CTF to sites engineered near the simian virus 40 replication origin stimulated replication by affecting the phasing of nucleosomes such that the origin remained nucleosome free have been performed (6). Transcription factors may also influence DNA replication initiation in a manner similar to the way they are believed to influence transcription, that is, by interacting with the replication machinery via direct or indirect protein-protein interactions. This is the case for NFI/CTF in adenovirus DNA replication; direct interactions between NFI/CTF and Ad Pol are believed to correctly position the viral replication machinery at the origin (2, 5, 31, 32). Results recently described by Verrijzer et al. (48) suggest that NFIII/OCT-1 may stimulate adenovirus DNA replication by the induction of DNA bending induced by binding at the site. Coenjaerts et al. have also shown that the spacing of the NFIII/OCT-1 site relative to the viral terminus is important for activity (8). It is difficult to understand how sequences outside the ITR are functionally redundant with the NFIII/OCT-1 site in our studies, since a linker replaces the NFIII/OCT-1 site in mutant *dl309-44/195* that would be expected to position the redundant, flanking elements at a distance where NFIII/OCT-1 does not function *in vitro*. The observation that the SP1 site adjacent to the NFIII/OCT-1 site also contributes to the efficiency of viral DNA replication (*dl309-53/195*; Fig. 3) supports the idea that NFIII/OCT-1, SP1, and the redundant elements between nt 106 and 194 may function similarly to the NFI/CTF sites when introduced adjacent to the simian virus 40 origin of replication (6); that is, by blocking the assembly of chromatin or the binding of other DNA-binding proteins at the adenovirus terminus, these binding activities may allow the adenovirus replication complex more ready access to the viral origin.

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